Protoporphyrin IX-dependent photodynamic production of endogenous ROS stimulates cell proliferation

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ABSTRACT

Photodynamic therapy using methyl 5-aminolevulinate (MAL) as a precursor of the photosensitizing agent protoporphyrin IX is widely used in clinical practice for the treatment of different pathologies, including cancer. In this therapeutic modality, MAL treatment promotes the forced accumulation of the endogenous photoactive compound protoporphyrin IX in target malignant cells. Subsequent irradiation of treated tissues with an appropriate visible light source induces the production of reactive oxygen species (ROS) that, once accumulated above a critical level, promote cell death. Here we demonstrate that a photodynamic treatment with low MAL concentrations can be used to promote a moderate production of endogenous ROS, which efficiently stimulates cell growth in human immortalized keratinocytes (HaCaT). We also show that this proliferative response requires Src kinase activity and is associated to a transient induction of cyclin D1 expression. Taken together, these results demonstrate for the first time that a combination of light and a photoactive compound can be used to modulate cell cycle progression through Src kinase activation and that a moderate intracellular increase of photogenerated ROS efficiently stimulates cell proliferation.

Keywords: methyl aminolevulinate, protoporphyrin IX, reactive oxygen species (ROS), photodynamic therapy, cell proliferation, cyclin D1, Src kinase.

INTRODUCTION

Photodynamic therapy (PDT) is an efficient clinical approach for the treatment of different types of cancer and other pathologies (Juarranz et al., 2008, Dolmans et al., 2003). This therapeutic modality is based on the combination of three elements: light, oxygen and a photosensitizer (PS). Once incorporated into target cells and during irradiation with an adequate light source, the PS absorbs photons of a specific wavelength and transfers the excitation energy to the molecular oxygen contained in the cell, giving rise to a range of highly reactive oxygen species (ROS), mainly singlet oxygen ($^1O_2$) (Dolmans et al., 2003, Juarranz et al., 2008). Raising ROS levels above a critical threshold irreversibly results in cell death (Buytaert et al., 2007, Oleinick et al., 2002). A critical step for an efficient PDT is the selective or enhanced accumulation of the PS in target cells. This process mainly relies on the specific chemical properties of the PS and on the altered metabolism of target cells in relation to the chemical properties of the PS (Juarranz et al., 2008). 5-Aminolevulinic acid (ALA) and its derivative methyl aminolevulinate (MAL) are the photosensitizing precursor agents most widely used in clinical practice (Kloek and Beijersbergen van, 1996, Kennedy et al., 1990, Casas and Batlle, 2002). These compounds are not PSs *per se* but metabolic precursors of the natural PS, protoporphyrin IX (PpIX). Incorporation of ALA or MAL to mammalian cells promotes the entry of both compounds in the heme biosynthetic pathway, resulting in an increased intracellular accumulation of PpIX susceptible to act as an endogenous PS (Kloek and Beijersbergen van, 1996, Kennedy et al., 1990, Casas and Batlle, 2002).

Experimental evidence obtained in the last years suggests that eukaryotic cells produce small amounts of ROS as part of different signal transduction pathways involved in cell survival and proliferation (Bartosz, 2009, Droge, 2002, Thannickal and Fanburg, 2000, Kamata and Hirata, 1999). Although the basic molecular mechanisms underlying these effects are largely unknown, it is assumed that most physiological effects of ROS are exerted through their interaction with critical intracellular thiols in cysteine residues of different proteins (Winterbourn and Hampton, 2008, Kamata and Hirata, 1999, Thannickal and Fanburg, 2000). Of particular importance are protein phospatases belonging to the cysteine-dependent phosphatase family, including classical protein tyrosine phosphatases (PTPs) and the lipid phosphatase PTEN, which have a critical cysteine residue in the catalytic site that is reversibly modified by fluctuating ROS concentrations. Thus, phosphatase function can be regulated by a delicate redox balance between ROS and the intracellular thiol pool (Chiarugi and Cirri, 2003, Ostman and Bohmer, 2001, Winterbourn and Hampton, 2008). This fact has been demonstrated for PTP inhibition induced by ROS during cellular signalling from receptor tyrosine kinases (Lee and Esselman, 2002, Chiarugi and Cirri, 2003). In this context, recent results indicate that Src family kinases are also redox-regulated proteins (Giannoni et al., 2010). Src kinases function can be regulated by ROS either indirectly, through inhibition of specific PTPs, or directly, through redox-dependent modification of several cysteine residues that can constitutively activate protein kinase function (Giannoni et al., 2010). It is well known that Src family kinases are key homeostatic modulators in different tissues and physiological situations through the regulation of numerous cellular

functions, including cell growth and survival, cell shape, adhesion and motility (Yeatman, 2004). The relevance of Src family kinases for the cellular physiology highlights the potential applicability of a regulatory mechanism acting on these proteins by a controlled modulation of intracellular ROS concentrations.

Here we have used immortalized human keratinocytes to explore the ability of PDT with MAL to stimulate cell growth *in vitro* in a finely tuned way. Our results show for the first time that PDT with MAL can efficiently induce cell proliferation through a tightly controlled and reproducible process that requires Src kinase activity and cyclin D1 expression and that it is not associated to genetic or cytotoxic damage.

MATERIALS AND METHODS

Cell culture and photodynamic treatments

HaCaT immortalized human keratinocytes were grown in Dulbecco´s Modified Eagle´s Medium (DMEM, Gibco) supplemented with bovine foetal serum (FBS 10%, Gibco), and 0.5% antibiotics (penicilline G [10000 U/ml] and streptomycin sulphate [10000 μg/ml] (Gibco) at 37ºC in a 5% CO_2 atmosphere.

Photodynamic treatments (PDT) were routinely performed on exponentially growing cells at 60%-70% confluence. Stock solutions of methyl 5-aminolevulinate hydrochloride 98% (MAL; Sigma) 10^{-2} M were prepared in distilled water. Cells were incubated for 5 hours with different working solutions of MAL (0.1 mM, 0.5 mM and 1 mM) and then irradiated for 10 minutes with a red light emitting diode (24 x 16 = 384 LEDs; WP7143 SURC/E) source with an emission peak at 634 nm and ± 20 nm bandwidth. The irradiance at the cell culture position was 6.2 mW/cm^2. Total light dose to the cultures was 3.72 J/cm^2. After treatments, fresh DMEM was added to the cells.

Quantification of protoporphyrin IX biosynthesis and intracellular ROS production

Cells growing at 50%-60% confluence were incubated for 5 hours with different MAL concentrations, collected and fixed with 3.7% aqueous formaldehyde solution for 15 minutes at room temperature (RT). Once washed and resuspended in PBS, cells were assayed for PpIX fluorescence in a flow cytometer (Cytomics FC500, Beckman Coulter) exciting at 620 nm and recording at 670 nm. 20.000 cells were measured for each condition (Uehlinger et al., 2000). Also, living cells on glass coverslips (Menzel-Gläser) were

incubated with different MAL concentrations and evaluated for PpIX emission (λ_{exc}= 546 nm) using a fluorescence microscope (Olympus BX-UCB).

Quantification of ROS production was performed as described (Daghastanli et al., 2008). After 5 hours of MAL treatment, 2,7-dichloro-dihydrofluorescein diacetate (DHF-DA; Fluka) was added to cell cultures to a final concentration of 10^{-5} M and incubated 1 hour. Cells were then subjected to irradiation as described, trypsinized and fixed with a 3.7% formaldehyde solution in PBS for 15-20 minutes at RT. ROS levels were evaluated by flow cytometry, exciting the oxidized dichloro-fluorescein (DCF) product with the 488 nm Ar^{+} laser line and measuring emission between 500 and 600 nm. 20.000 cells were measured for each condition. Experiments were done in parallel on cell cultures treated with different MAL concentrations, then with 10^{-5} M DHF-DA, and observed in a fluorescence microscope for ROS detection through DCF emission (λ_{exc}= 436 nm).

Cell growth quantification

Cells grown in 24-well plastic plates were incubated with MAL and irradiated as indicated above. Cell cultures were subjected to MTT viability assays at different times (24, 48, 72 or 96 hours) after PDT. Briefly, cells were incubated for 2 hours with MTT (50 μg/ml) in DMEM, DMSO was added to solubilize the produced formazan and the optical density at 542 nm was measured with a SpectraFluor (Tecan).

For mitotic index analysis, cell cultures subjected to PDT were fixed with cold methanol (-20º C), stained for 5 minutes in a 2 μg/ml solution of Hoechst 33258 (H-33258, Sigma), washed in water, air dried and mounted in DePeX (Serva). Samples were evaluated under fluorescence microscopy using UV

excitation (365 nm). Mitotic index (MI) was determined as the % number of cells in division. About 1000 cells were counted for each point for MI.

For cell cycle analyses cells were subjected to PDT as described above. After 24 hours, cells were trypsinized and fixed in 70% ethanol for 30 minutes at -20º C, washed in PBS and stained with a cell cycle assay kit (Immunostep), which incorporates RNAse and propidium iodide (PI). The samples were evaluated in the flow cytometer by exciting at 488 nm, comparing with appropriate control cells.

For quantification of cyclin D1 protein levels, exponentially growing cells were subjected to PDT. Non-irradiated cells were used as control conditions. After the PDT, cells were further grown for different times, collected and fixed with 3.7 % formaldehyde solution for 15 minutes at RT. Cells were treated for 30 minutes with a 0.1% Triton X-100, washed, collected and incubated for 1 hour with an antibody against cyclin D1 (mouse monoclonal anti-cyclin D1, NovoCastra) at 37º C. Cells were washed in PBS, collected and incubated for 45 minutes with a secondary antibody (goat anti-mouse IgG-FITC conjugate, Sigma) at 37º C. Cells were washed and resuspended in PBS and cyclin D1 expression level was measured by flow cytometry using a 488 nm excitation line. 20.000 cells were counted in each experiment.

Genotoxic damage quantification

For the evaluation of genotoxic damage, cells were grown on coverslips, subjected to different photodynamic treatments, and fixed with 3.7% formaldehyde in PBS. The TUNEL assay (Gavrieli et al., 1992) was used to determine the appearance of strand breaks in the DNA molecule after PDT with MAL. Cell samples were processed following the instructions of the

manufacturer (Roche). The formation of phosphorylated histone H2AX (pH2AX) foci on the chromatin fibre in response to genotoxic injury (Yuan et al., 2010) was determined by immunofluorescence or immunoblot analysis using a specific mouse monoclonal anti-phosphoH2AX and H3 antibodies (Abcam) following described procedures (Espada et al., 2009). The % of positive cells for either TUNEL or pH2AX assays after PDT with different MAL concentrations was quantified for a sample size of 100-200 cells in two different experiments.

Inhibition of Src kinase and NOX activities and depletion of intracellular ROS accumulation

The specific Src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2; Sigma) (Zhang et al., 2008) was used at 5 μM in DMEM; the NOX inhibitor diphenyleneiodonium chloride (DPI; Sigma) (Ranjan et al., 2006) was used at 1 μM in DMEM. Cells were incubated with both compounds for 5 hours and subjected to PDT with MAL. Stock solutions of both compounds were made in DMSO. Cell cultures treated with DMSO were used as control samples.

To remove intracellular ROS produced during PDT, cells were treated with the antioxidant singlet oxygen scavenger 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) at 10^{-3} M for 1 hour before PDT.

Statistical analysis

The numerical treatment of results was done with the SPSS 15.0 software package (SPSS Inc., IBM, Chicago, IL, USA). Both t-Student and Analysis of Variance (ANOVA) tests were employed to evaluate statistical significance of results.

RESULTS

Moderate PpIX-dependent intracellular ROS photogeneration efficiently stimulates cell proliferation

We have previously established two PDT protocols for skin keratinocytes designed to obtain different and defined cellular responses: a lethal condition (1 mM photosensitizer) that promotes a fast and severe cell death response by necrosis and a sublethal condition (0.5 mM photosensitizer) that promotes a delayed cell death response after activation of an E-cadherin dependent apoptotic program (Espada et al., 2009, Galaz et al., 2005). Here we have tested a milder condition, 0.1 mM MAL, to evaluate potential photoactivating effects of PDT on cell proliferation.

First we analyzed the production rate of the endogenous photosensitizer PpIX after MAL treatments at different concentrations and the photoproduction rates of ROS after PDT using MAL at the indicated concentrations. When PpIX production was measured, cell cultures were exposed to MAL but were not subsequently irradiated with red light, preventing both photodynamic damage and PpIX photobleaching. The characteristic red fluorescent emission under 546 nm light irradiation was used to quantify PpIX production. For ROS production quantification, cells were exposed to MAL and then irradiated with red light to induce the photodynamic action and subsequent photodamage. The oxidized DCF fluorescent probe was used to trace ROS formation. Intracellular ROS localization and PpIX rate of synthesis were evaluated in fixed samples by flow cytometry, as well as by fluorescence microscopy in living cells after different MAL treatments.

As expected, we found a correlative MAL-dose dependent production of PpIX and ROS in HaCaT cells after PDT. A strong induction of both compounds was observed after 0.5 and 1 mM MAL treatments (Fig. 1A). Interestingly, a moderate but statistically significant increase of endogenous ROS levels was also observed after 0.1 mM treatments (Fig. 1A). In agreement with these observations, we observed that, as compared to non-treated cells, a low and diffuse cytoplasmic PpIX red signal was detected in HaCaT cells after MAL 0.1 mM conditions which strongly increased in the cytoplasm and accumulated in the cell membrane in cells after MAL 0.5 or 1 mM conditions (Fig. 1B and data not shown). Equivalent results were found for DCF-dependent localization of ROS production, higher MAL concentrations promoting a higher DCF signal (Fig. 1B). It is to note that, as expected, cells used for ROS quantification showed morphological alterations associated with severe (1mM MAL) PDT photodamage. Phase-contrast microscopy analysis revealed that cell morphology was virtually unaffected in 0.1 mM MAL treated cells while it was severely disturbed after 0.5 or 1 mM MAL treatments, showing prominent morphological characteristics of cell death induction (Fig. 1B). These observations indicate that low MAL concentrations (0.1 mM) promote a low accumulation of PpIX that is sufficient to induce a moderate ROS synthesis after PDT in the absence of cell death.

We next evaluated the biological potential of the moderate ROS increase induced by PDT with MAL 0.1 mM treatment to modulate cell proliferation in HaCaT cells. We found that this low increase of intracellular ROS levels promoted by 0.1 mM MAL phototreatments was sufficient to induce a significant and reproducible increment of the mitotic index in HaCaT cells (Fig. 1C)

associated to an acute increase of the S-phase cell population 24 hours after the treatment (Fig. 1D). No mitogenic effects were observed when cells were treated with MAL in the absence of red light or with red light in the absence of MAL (Fig. 1C, D). Notably, we have also found that the mitogenic effect of PDT with MAL on HaCaT cells was strongly enhanced by a two-step sequential photodynamic treatment (Fig. 2). Thus, a second 0.1 mM MAL treatment 48 hours after the first one resulted in a statistically significant increase at 72 hours of a roughly 10% in the number of viable cells as compared to samples stimulated by a single treatment (Fig. 2). It is to note that in this experiment the mitotic index ratio decreased between 24 and 48 hours while the number of viable cells increased (Fig. 2). However, although the mitotic index ratio decreased between 24h and 48 hours, it actually did not returned to 0 values. Instead, it steadily returned to a background level in which the mitotic ratio of treated samples is the same as that of non-stimulated, normal proliferating controls. This implies that, between 24 and 48h, PDT treated samples still present higher mitotic ratios than controls. In these conditions, given a previously higher number of viable cells and a higher mitotic ratio as compared to controls, a steady increase in the number of viable cells in treated samples was observed (Fig. 2). In this context, our results indicate that a second PDT treatment at 48 hours can hold back the declining trend of the mitotic ratio, promoting a new increase at 72 hours, which result in a concomitant and additional increase in the number of viable cells (Fig. 2). As a whole, these results demonstrate that a combination of red light and increased levels of PpIX, resulting in a moderate production of intracellular ROS, can be used to stimulate cell growth.

PpIX-dependent photodynamic stimulation of cell proliferation does not promote genotoxic or cytotoxic damage

Treatment of cultured cells with an exogenous ROS source, such as H_2O_2, can promote a moderate increase of cell proliferation but also extensive and/or irreversible cell damage (Pryor et al., 2006). To discard deleterious effects induced by PDT with 0.1 mM MAL, we investigated potential genotoxic or cytotoxic damage using different approaches.

We first evaluate the incidence of PDT with MAL on cell viability using the MTT assay. We found no significant effects of PDT with 0.1 mM MAL on cell survival at either the standard 10 minutes red light irradiation time or at longer irradiation times up to 30 minutes (Fig. 3A). By contrast, PDT with 0.5 or 1 mM MAL resulted in a significant cytotoxic effect with 10 minutes irradiation time, greatly increased with longer irradiation times (Fig. 3A). This result is in close agreement with our observations regarding the effects on cell morphology of PDT with different MAL concentrations (Fig. 1B).

We next analyzed potential genotoxic effects of PDT with 0.1 mM MAL by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect DNA breaks (Gavrieli et al., 1992) or by detection of extended phosphorylated histone H2AX (pH2AX) foci formation on the chromatin fibre associated to severe DNA damage (Yuan et al., 2010). Interestingly, a strong increase of pH2AX nuclei was observed by immunofluorescence and immunoblot analysis in apoptotic 0.5 mM MAL treated cells, but not in viable 0.1 mM MAL treated cells or necrotic 1 mM treated cells (Fig. 3B, C, D). This result demonstrate that the pH2AX signal of genomic damage was triggered by 0.5 mM sub-lethal treatments but not by stimulating 0.1 mM conditions and

indicated that the necrotic nuclei caused by 1 mM treatments were also unable to trigger a pH2AX-dependent response to genomic damage. Likewise, our results showed that, as expected, PDT with 0.5 or 1 mM MAL induced a strong DNA fragmentation revealed by TUNEL assay (Fig. 3B, D). In sharp contrast, no such damaging effects were observed after PDT with 0.1 mM MAL (Fig. 3B, D). As a whole, these results indicate that PDT with 0.1 mM MAL is a cellular-safe process to stimulate cell proliferation in the absence of cytotoxic or genotoxic side effects.

PpIX-dependent photodynamic stimulation of cell proliferation requires ROS production and it is associated with an increase in cyclin D1 expression

To elucidate the molecular basis underlying the stimulation of cell proliferation by PDT with MAL, we first analyze the requirement of ROS in this process. To this end, cells subjected to PDT with 0.1 mM MAL were treated during light irradiation with the antioxidant DABCO, a known $^1O_2$ scavenger that efficiently blocks intracellular effects of ROS increase. We found that DABCO completely inhibited the stimulatory effect of PDT with MAL on cell proliferation without noticeable effects on cell viability (Fig. 4A). This result demonstrates that photostimulation of cell proliferation with MAL mainly relies on a moderate increase of intracellular ROS.

We hypothesized that the increase of the mitotic index and the induction of cell proliferation after PDT with MAL was necessarily associated to a concomitant expression of cell cycle regulatory proteins. To corroborate this idea we carried out a time course analysis of cyclin protein expression after cell photoactivation with MAL. In particular, we found a strong and transient

increase of cyclin D1 protein levels between 3 to 8 hours after PDT with MAL in HaCaT cells (Figure 4B). The expression of cyclin D1 induced after PDT with MAL was completely inhibited by intracellular ROS scavenging with DABCO. These results indicate that cell proliferation induced by PDT with MAL is associated to a transient increase in cyclin D1 expression which depends on ROS production.

PpIX-dependent photodynamic stimulation of cell proliferation and transient cyclin D1 expression depends on Src kinase activity

It is well known that Src kinases can regulate cell cycle progression through activation of cyclin D1 expression (Sinibaldi et al., 2000, Leslie et al., 2006). It has also become clear in the last years that Src kinases are regulated by intracellular ROS (Giannoni et al., 2010). In this context, we reasoned that the activity of Src kinase was probably implicated in the stimulation of cell proliferation and in the concomitant transient activation of cyclin D1 after PDT with 0.1 mM MAL. To evaluate this hypothesis, we performed a series of photoactivation experiments using our established MAL-dependent conditions to stimulate cell proliferation in the presence or absence of the specific Src kinase inhibitor PP2. Our results clearly showed that, as compared to control samples, Src kinase activity inhibition had no effect on cell viability but significantly blocked the induction of cell proliferation promoted by PDT with 0.1 mM MAL (Fig. 5A). In the same way, we also observed that Src kinase inhibition completely aborted the transient cyclin D1 expression that we had observed to be associated to PDT with 0.1 mM MAL (Fig. 5B). Taken as a whole, these results suggest that cell proliferation stimulation after PDT with 0.1 mM MAL on

HaCaT cells is a direct result of a transient expression of cyclin D1 induced by Src kinase activity.

The mitochondria are important foci of intracellular ROS production. However, mitochondrial ROS production has mainly to do either with inefficient electron leakage to molecular oxygen during aerobic respiration, or with specific signaling for the initiation and promotion of apoptotic processes. It is now widely accepted that NOX activity is the main source of physiological ROS involved in cell signaling through interaction with specific components of cell signaling pathways (Nohl et al., 2005, Raddatz et al., 2011, Brown and Borutaite, 2011) . In this context, we also wanted to evaluate the effect of NOX enzymatic inhibition on the stimulatory effect of PDT with 0.1 mM MAL. To this end, we used the NOX inhibitor DPI in PDT with 0.1 mM MAL experiments. Our results showed that, as compared to control samples, inhibition of NOX activity did not affected neither cell proliferation or viability, nor cyclin D1 expression induced by PDT with 0.1 mM MAL (Fig. 5A, B), indicating that the production of endogenous ROS in this process is independent of NOX activity.

DISCUSSION

The possibility to stimulate cell proliferation using light and an endogenous photoactive compound is a provocative approach that can have a broad range of applications from biotechnology to regenerative medicine. In this study we report the stimulation of cell proliferation in immortalized human keratinocytes after a mild photodynamic treatment which is strongly enhanced by a sequential two-step protocol. We have demonstrated that the induction of cell proliferation by PDT with MAL is mediated by singlet oxygen production induced by PpIX photodynamic activation; this cell stimulation is associated to a transient upregulation of cyclin D1 expression and depends on Src kinase activity.

The Cyclin family of cell cycle regulator proteins has been identified as an important target in the redox-dependent regulation of cell cycle progression. In particular, it is known that exogenous ROS can regulate cyclin D1 levels through the modulation of classical tyrosine phosphatase activity (Ranjan et al., 2006, Martinez Munoz et al., 2001, Burch and Heintz, 2005) and can also modify the proteasome-dependent metabolic degradation balance of cyclin D1 through a redox-dependent pathway (Martinez Munoz et al., 2001). In the same way, it has been reported that overexpression of catalase decreases the transition from G_1 to S in a cyclin D1-mediated mechanism (Onumah et al., 2009). In agreement with these observations, the results presented here indicate that a moderate endogenous ROS production can rapidly promote a transient increase in cyclin D1 expression. As a whole, these observations point to the evidence of an extended redox mechanism for the synchronized generation of ROS during cell cycle in mammalian cells that is implicated in the

regulation of cyclin D1 expression (Menon and Goswami, 2007, Burch and Heintz, 2005, Takahashi et al., 2004).

It has been shown that exposure of different mammalian cell types to an exogenous source of ROS results in the phosphorylation and activation of large number of signalling molecules implicated in cell cycle regulation, including receptor tyrosine kinases, PKC, PKB, Src kinase PLC1γ and MAPKs (Boonstra and Post, 2004). In the same way, different studies indicate that different ligands, including TNFα, TGFβ1, interleukin1, PDGF and EGF can promote a receptor activation-dependent increase of ROS (Boonstra and Post, 2004). In this context, our results using a PDT approach can be considered as an additional proof of concept for the more general idea that a moderate and transient endogenous ROS production can stimulate cell proliferation (Menon and Goswami, 2007, Takahashi et al., 2004, Boonstra and Post, 2004).

At present, it is accepted that the main source of intracellular ROS for cell signalling is the result of the activity of the NADPH-oxidase (NOX) protein family (Bedard and Krause, 2007, Chan et al., 2009). Here we have shown that stimulation of cell proliferation by PDT with MAL requires ROS production but it is independent of NOX activity. Since the main ROS source during PDT with MAL is PpIX, this is an expected result and reinforces the view that regulating the biosynthetic production of PpIX by dosing the given amount of MAL is a valuable tool to modulate the production of endogenous ROS to obtain different cellular responses. On the other side, our results have also shown that stimulation by PDT with MAL entirely depends on Src kinase activity. Taking into account that Src kinase can be constitutively activated by intracellular ROS (Giannoni et al., 2010) and that cyclin D1 is a target of Src kinase activity (Leslie

et al., 2006, Yeatman, 2004, Sinibaldi et al., 2000), our results reinforce the emerging concept of the regulation of intracellular ROS balance as an important cell signalling mechanism.

In conclusion, the results reported here demonstrate for the first time that red light in combination with an appropriate photosensitizer can be used to manipulate the cell cycle activity at a specific point by the punctual photogeneration of signaling ROS. As a rule, eukaryotic cells present the least reduced cytoplasmic environment during the first half of the G_1 phase of the cell cycle (Winterbourn and Hampton, 2008). During this period there exists an optimum ROS stimulating window, during which the cell can efficiently respond to peaks of transient endogenous ROS production. Once the cell is committed to enter the cell cycle, mainly through cyclin D1 upregulation, the cytoplasm adopts a reducing trend in its redox status that makes it refractory to any further ROS signaling until the cell completes the M phase (Menon and Goswami, 2007). In this context, it can be hypothesized that photostimulation of cell proliferation after mild MAL treatments specifically affects cells that are in the early and middle G_1 phase. This experimental approach opens the possibility to photostimulate tissues to improve its homeostatic response in processes such as wound healing and organ repair.

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FIGURE LEGENDS

Figure 1. A protoporphyrin IX-dependent moderate increase of intracellular ROS stimulates cell proliferation in HaCaT cells. A) Quantification by flow cytometry of protoporphyrin IX (PpIX) accumulation and ROS production after PDT with MAL at different concentrations. The characteristic red emission under 488 nm exciting light was used to measure PpIX production. 2,7-dichloro-dihydrofluorescein diacetate probe was used to measure ROS formation. The data are the average values of three independent experiments and the mean ± SD values are represented. B) Representative fluorescent microscopy images showing PpIX production after MAL at different concentrations or ROS production after PDT with MAL at different concentrations. Phase contrast microscopy was used to evaluate cell morphology. Bar: 20 μm. C) Measurement of the mitotic index in cells treated with 0.1 mM MAL and irradiated with red light, cells treated with MAL but not irradiated, and cells that were neither treated with MAL nor irradiated (Control). Results were obtained 24 hours after treatments. The data are the average values of three independent experiments and the mean ± SD values are represented. D) Cell cycle analysis (24 hours) by flow cytometry of cells treated with 0.1 mM MAL and corresponding controls. The arrowhead indicates a strong increase of the S-phase population in 0.1mM MAL + light treated cells. Results are representative of three independent experiments.

Figure 2. A sequential two-step photodynamic treatment with MAL significantly enhances the stimulation of cell proliferation. Quantification of the number of viable cells (A) and the mitotic index (B) in cells treated with a single round of PDT with 0.1 mM MAL at time 0 hours as compared to cells subjected to a second round at time 48 hours. Results are represented as the ratio of the % of positive (viable or mitotic) cells measured in 0.1 mM MAL + light (irradiated) samples with respect to 0.1 mM MAL (non irradiated) samples. Grey arrows indicate the time point of phototreatments. The data are the average values of three independent experiments and the mean ± SD values are represented.

Figure 3. Stimulation of cell proliferation after photodynamic treatment with MAL it is not associated to cytotoxic or genotoxic damage. A) Quantification of cell viability 24 hours after photodynamic treatment with different MAL concentrations and at different red light irradiation times. (B) Determination by fluorescence microscopy of histone pH2AX (upper panels) or TUNEL (lower panels) positive cells after photodynamic treatments with different MAL concentrations. Bars: 10 μm. (C) Evaluation of protein expression levels by immunoblot of phosphorylated histone H2AX (pH2AX) and histone H3. Results are representative of two different experiments. (D) Quantification of the % of pH2AX or TUNEL positive cells after photodynamic treatments with different MAL concentrations. 100-200 cells for each condition were evaluated in two independent experiments and the mean ± SD was represented.

Figure 4. Stimulation of cell growth after photodynamic treatment with MAL depends on endogenous ROS production and it is associated to a transient increase in cyclin D1 expression. A) Quantification of mitotic index and cell viability after 24 hours in cells treated with 0.1 mM MAL and irradiated with red light as compared to non-irradiated cells treated with 0.1 mM MAL in the presence or absence of the singlet oxygen scavenger DABCO. The data are the average values of three independent experiments and the mean ± SD values are represented. B) Time-course quantification by flow cytometry of cyclin D1 expression levels associated to photodynamic treatment with MAL in the presence or absence of DABCO. Results are represented as the ratio of cyclin D1 expression measured in 0.1 mM MAL + light (irradiated) samples with respect to 0.1 mM MAL (non irradiated) samples. The data are the average values of three independent experiments and the mean ± SD values are represented.

Figure 5. Stimulation of cell growth after photodynamic treatment with MAL depends on Src kinase activity. A) Quantification of mitotic index and cell viability after 24 hours in cells subjected to photodynamic treatment with MAL in the presence or absence of the NOX inhibitor DPI or the specific Src kinase inhibitor PP2. Results are represented as the ratio of the % of positive (mitotic or viable) cells measured in 0.1 mM MAL + light (irradiated) samples

with respect to 0.1 mM MAL (non irradiated) samples. The data are the average values of three independent experiments and the mean ± SD values are represented. B) Time-course quantification by flow cytometry of cyclin D1 expression levels associated to photodynamic treatment with MAL in the presence or absence of DPI or PP2. Results are represented as the ratio of cyclin D1 expression measured in 0.1 mM MAL + light (irradiated) samples with respect to 0.1 mM MAL (non irradiated) samples. The data are the average values of three independent experiments and the mean ± SD values are represented.

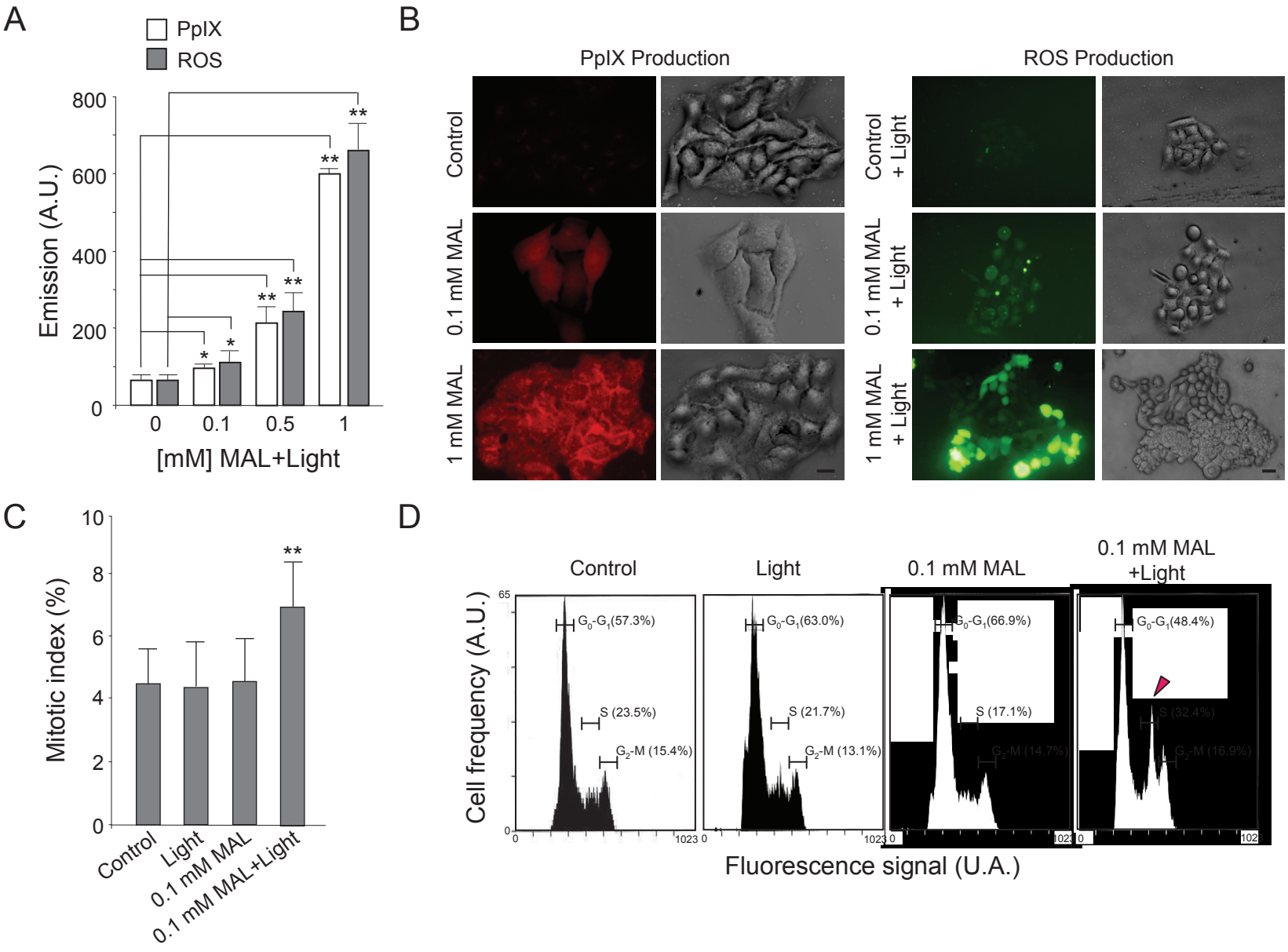

Figure 1. Blázquez-Castro et al. 2011

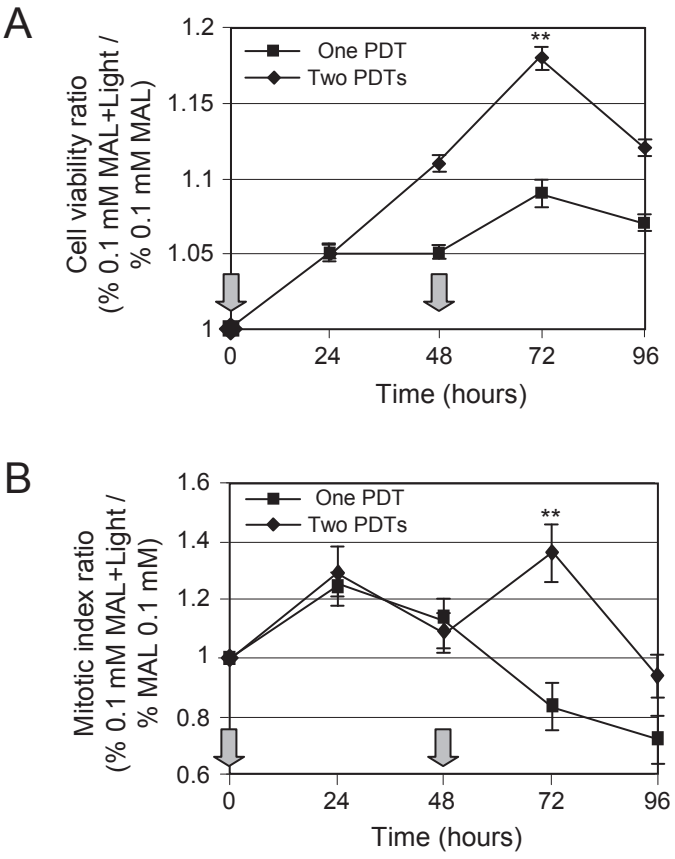

Figure 2. Blázquez-Castro et al. 2011

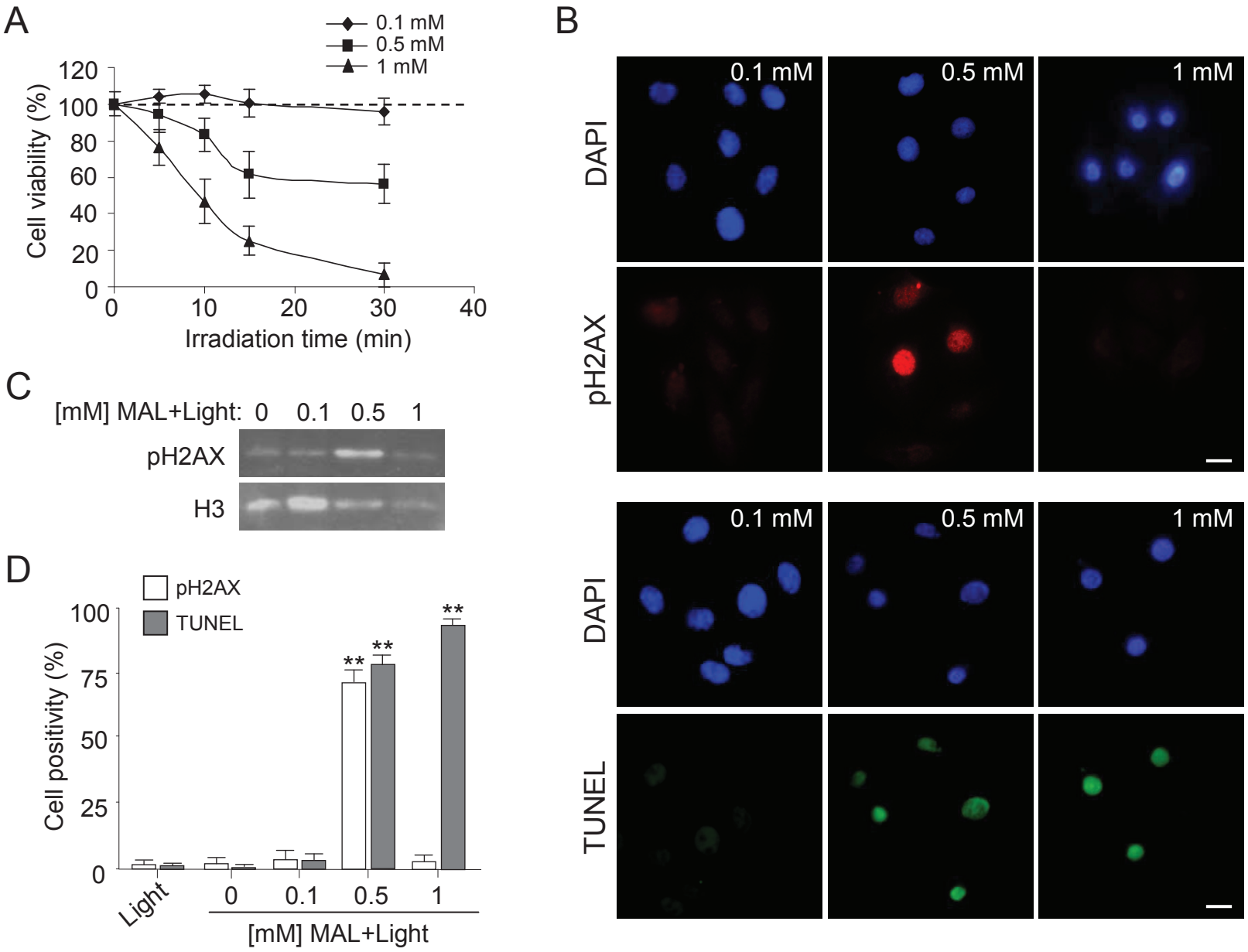

Figure 3. Blázquez-Castro et al. 2011

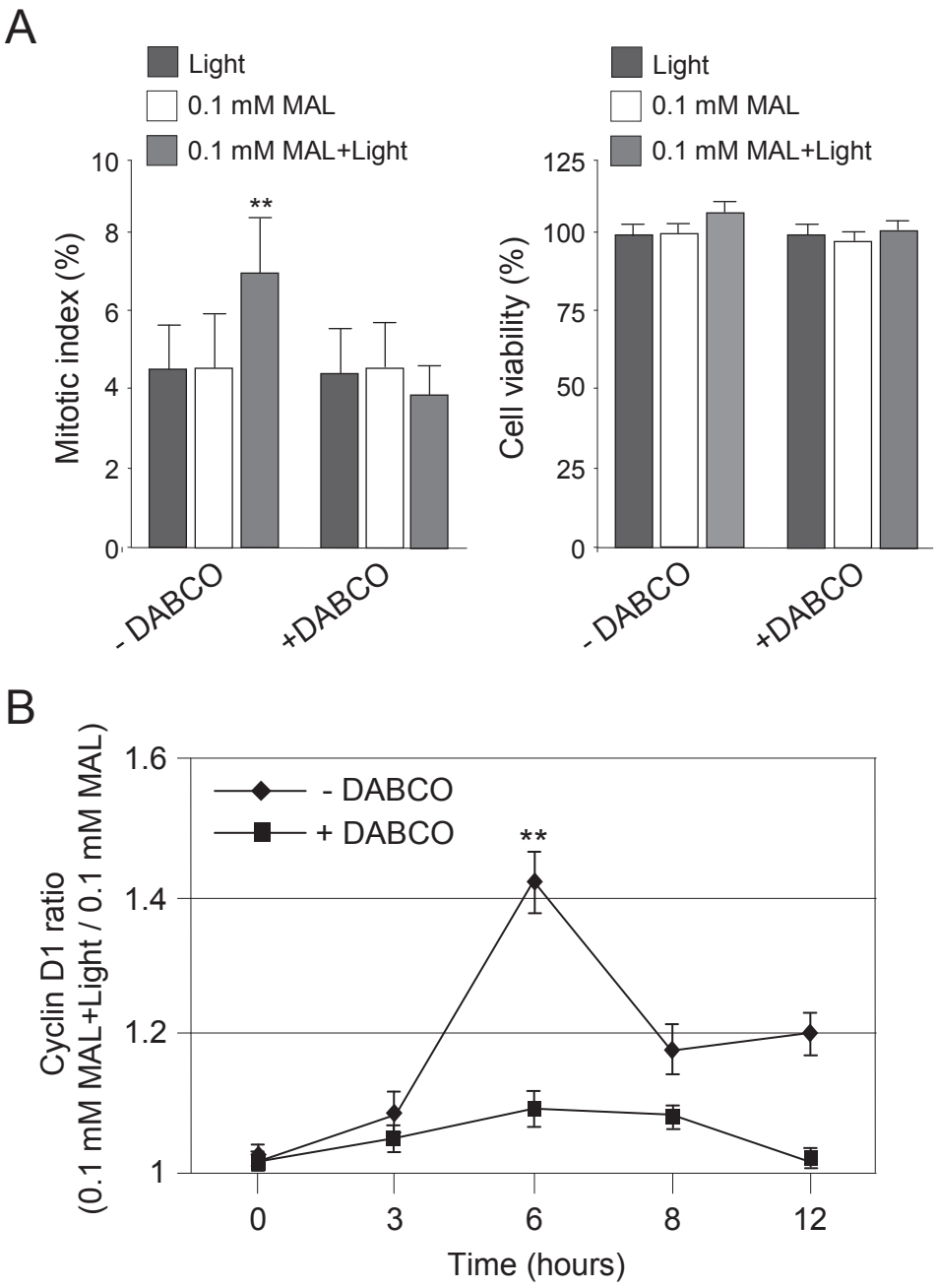

Figure 4. Blázquez-Castro et al. 2011

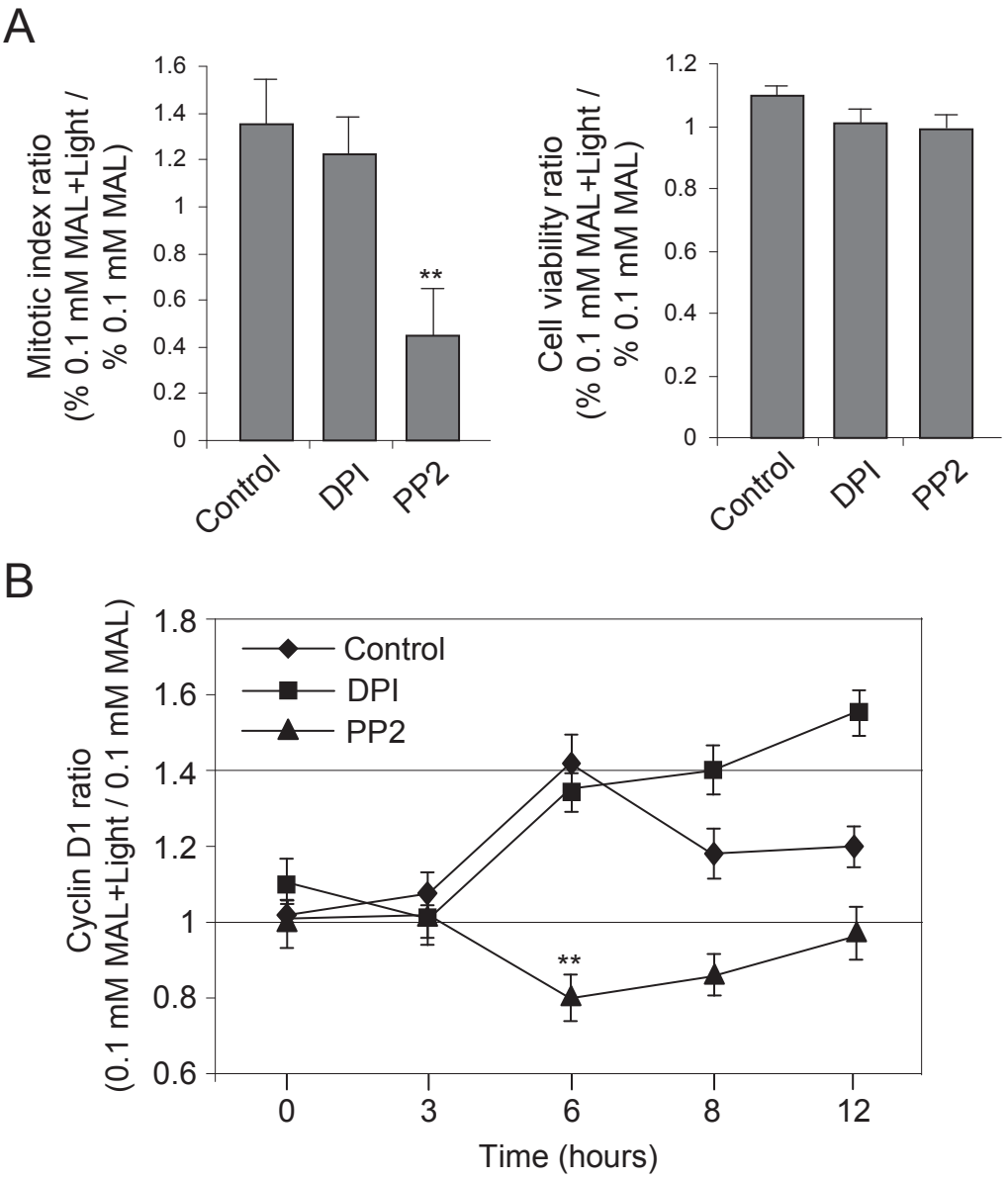

Figure 5. Blázquez-Castro et al. 2011